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<b>13. ABSTRACT (Maximum 200 Words)</b> We established for the first time that an oral DNA minigene vaccine encoding muring vascular endothelial growth factor receptor-2 (FLK-1)-derived nonapeptides with H-2D <sup>b</sup> anchor residues can evoke a CD8 <sup>+</sup> cytotoxic T-cell response. These CTLs effectively kill proliferating endothelial cells in the neovasculature of RM-9 murine prostate carcinoma cells leading to the effective suppression of tumor growth and hepatic as well as pulmonary metastases in syngeneic C57BL/6J mice. These data strongly support the contention that the murine vascular endothelial growth factor receptor-2 (FLK-1), overexpressed on proliferating endothelial cells in the tumor vasculature, is an effective target for DNA-minigene vaccines against prostate cancer.				
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## INTRODUCTION

The overall objective remains the same, namely suppression of tumor angiogenesis resulting in the ablation of murine prostate tumors and their metastases in syngeneic C57BL/6J mice. This will be accomplished with oral DNA minigene vaccines derived from the murine VEGF-receptor 2 (FLK-1) consisting of FLK-1 nonapeptide minigenes with either H-2D<sup>b</sup> or H-2K<sup>b</sup> anchors in combination with CD40 ligand trimer (CD40LT). The initial focus is on demonstrating that an effective suppression of RM9 murine prostate tumor growth can be achieved by this DNA vaccine in syngeneic C57BL/6J mice. Second, it was important to demonstrate that a FLK-1 based minigene vaccine could effectively suppress both lung and liver metastases of RM9 prostate carcinoma and to specify which H-2 anchors were most effective in this regard. Third, it was critical to demonstrate that a FLK-1 peptide-specific CD8<sup>+</sup> T cell response was evoked against murine endothelial cells which expressed FLK-1 and to ascertain which peptide was effective in this regard. Finally, it was important to demonstrate that immunization with the FLK-1 minigene was truly effective since it induced an immune response characterized by activated T cells as indicated by their production of the proinflammatory cytokine IFN- $\gamma$ , both at the intracellular and single T cell levels. The data obtained during this grant period clearly indicate that we have produced a novel and unique DNA minigene vaccine which can evoke a robust CTL response that effectively suppresses growth and dissemination of prostate carcinoma in a mouse tumor model.

## BODY

Task 2: (Months 16-24) as outlined in the initial grant proposal and in the revised SOW was mostly completed. The hypothesis was validated that a FLK-1 based DNA minigene vaccine can effectively suppress growth and hepatic as well as pulmonary metastases of murine RM-9 prostate carcinoma in a syngeneic tumor model in C57BL6/J mice. This was achieved most effectively by the induction of a robust CD8<sup>+</sup> T cell response evoked by a polyubiquitinated DNA minigene vaccine with H-2D<sup>b</sup> anchors that also co-expressed CD40LT. The H-2D<sup>b</sup> peptide epitopes were fused with the HIV tat peptide to enhance immunogenicity. Experimental groups of mice immunized with controls such as empty vector (pUB) showed no tumor-protective response and vaccines that only encoded either FLK-1 or CD40LT also proved considerably less effective in evoking a tumor-protective immune response. Minigene vaccines encoding peptides with H-2K<sup>b</sup> anchor residues proved to be ineffective in this regard as did those encoding H-2D<sup>b</sup> peptides 1 and 3. In contrast the minigene vaccine encoding H-2D<sup>b</sup> peptide 2 was most effective not only in suppressing growth and metastases of RM-9 prostate carcinoma but also in evoking robust CD8<sup>+</sup> CTLs which effectively killed endothelial cells expressing FLK-1. This particular minigene vaccine also induced highly activated CD8<sup>+</sup> CTLs as evident from their production of proinflammatory cytokine IFN- $\gamma$  both at the intracellular and single T cell level.

The construction of the various FLK-1-derived minigenes are shown schematically in Fig.1. The suppression of RM-9 prostate tumor growth by both a DNA vaccine encoding the entire FLK-1 gene and by various H-2D<sup>b</sup> and H-2K<sup>b</sup> minigene vaccines is depicted in Fig. 2. The efficacy in suppressing both lung and liver metastases of RM-9 prostate carcinoma is clearly demonstrated for H-2D<sup>b</sup>-based DNA minigene vaccines in Fig. 3. The cytotoxicity induced by the various minigene vaccines and the vaccine encoding the entire FLK-1 gene is indicated in

Fig. 4 by the specific lysis produced against endothelial cells expressing FLK-1. The superior specific cytotoxic killing activity of CTLs induced by the H-2D<sup>b</sup> minigene encoding peptide 2 against endothelial target cells expressing FLK-1 is indicated since whole tumor cells lacking FLK-1 expression were not killed at all (Fig. 5). Finally, Fig. 6 clearly illustrates that the H-2D<sup>b</sup> minigene encoding peptide 2 is also most effective in inducing highly activated T cells as indicated by the production of IFN- $\gamma$  at both the intracellular level and the single T cell level.

## KEY RESEARCH ACCOMPLISHMENTS

We have generated a FLK-1 based DNA minigene vaccine with defined H-2D<sup>b</sup> anchor residues that co-expresses CD40LT and proved most effective in evoking a CD8<sup>+</sup> T cell response. This, in turn, resulted in the strong suppression of RM-9 prostate carcinoma tumor growth and disseminated liver and lung metastases.

## REPORTABLE OUTCOME

A manuscript which describes the results summarized here is currently in preparation.



## CONCLUSIONS

We could establish for the first time that a FLK-1 based DNA minigene vaccine is capable of evoking a CD8<sup>+</sup> T cell response sufficiently robust to induce the suppression of growth and metastases of murine RM-9 prostate carcinoma. Together with our previous studies on the DNA vaccine encoding the entire FLK-1 gene, these data strongly support the contention that the murine VEGF-receptor 2, overexpressed on proliferating endothelial cells in the tumor vasculature, is an effective target for a DNA vaccine against prostate carcinoma.

## APPENDIX

**Fig. 1 . Schematic Presentation of minigenes derived from FLK-1 for construction of oral DNA vaccines**

**Fig. 2. Suppression of RM-9 prostate tumor growth by FLK-1 and FLK-1 minigene vaccines.** (A) Suppression of prostate tumor growth in syngeneic C57BL/6J mice after immunization combined with CD40LT. Comparisons are depicted between (a) pUb; (b) pUb-FLK-1; (c) pCD40LT; and (d) pUb-FLK-1 + pCD40LT vaccines. Data are shown as mean lung weight ( $n = 6$ ); bars,  $\pm$  SD. The differences between experimental groups and controls are statistically significant  $P < 0.0001$ . (B). Induction of tumor-protective immunity by FLK-1 derived minigene vaccines. C57BL/6J mice ( $n = 8$ ) received three vaccinations by gavage of  $10^8$  double attenuated *Salmonella typhimurium*, harboring DNA vaccines at 2-week intervals. Experimental groups were immunized with vaccines encoding murine H-2Kb and Db peptide epitopes fused with HIV tat peptide to enhance their immunogenicity. Control groups of mice ( $n = 8$ ) included those treated with PBS (a) or a vaccine containing only the empty vector pCMV(b) or HIV tat (c). Experimental groups included those that received the H-2Kb minigene (HIV-tatK<sup>b</sup>) (d) the H-2Db minigene (HIV-tatD<sup>b</sup>) (e). Mice were challenged with  $1 \times 10^5$  RM-9 murine prostate carcinoma cells 1 week after the final vaccination. Bars represent means and standard deviations of eight mice per group. Differences in tumor weights between experimental groups vaccinated with the HIV-tatD<sup>b</sup> minigene and all control groups were statistically significant ( $P < 0.01$ ).

**Fig. 3. Demonstration of HIV-tatD<sup>b</sup> minigene vaccine responses against RM-9 prostate carcinoma in both lung and liver metastases models.** (A). Efficacy of the HIV-tatD<sup>b</sup> minigene in the experimental lung metastasis model. C57BL/6J mice were immunized 3 times followed by i.v. injection of  $5 \times 10^4$  RM-9 cells 1 week after the last immunization. Grossly visible metastases were determined on the surface of the organs 28 days after tumor cell injection, at which time, the animals were sacrificed and examined further for micro metastases. Lungs were weighed and tissue specimens of mice without visible metastases were stained with hematoxylin/eosin and examined histologically. (B). Efficacy of HIV-tatD<sup>b</sup> minigene against experimental liver

metastases. The immunization protocol is the same as that described above. Mice were challenged with RM-9 prostate tumor cells by injection with a 27 gauge needle beneath the splenic capsule, followed by ligation of the splenic pedicle. All animals were sacrificed and examined for metastases. The differences between the controls (pCMV and pHIV tat) or HIV-tatD<sup>b</sup> in either lung or liver metastasis models were statistically significant ( $p < 0.001$ ). The normal weights of lung and liver are from 0.2 to 0.3g and from 1 to 1.2 g, respectively.

**Fig. 4. Cytotoxicity induced by CD8<sup>+</sup> T cells of C57BL/6J mice against murine endothelial cells after immunization with DNA vaccines encoding FLK-1 derived vaccines.** Splenocytes were isolated 1 week after the third vaccination, and CD8<sup>+</sup> T cells analyzed for their lytic activity in a 4-h <sup>51</sup>Cr-release assay at different effector-to-target cell ratios (E:T). Specific lysis is indicated that was mediated by CD8<sup>+</sup> T cells from different experimental groups of mice: pCMV (◆), and HIV tat (□), HIV-tatD<sup>b</sup> (△), pUb-FLK-1 (○).

**Fig. 5. T-cell induced cytotoxicity against endothelial cells and RM-9 prostate carcinoma cells.** Three H-2D<sup>b</sup> peptides that were synthesized at the level of 95% purity were mixed with naïve T cells isolated from splenocytes of C57BL/6J mice and co-cultured for 3 days. The standard <sup>51</sup>Cr-release assay was then performed using MS1 endothelial cells (◆) and RM-9 tumor cells (■) as respective targets. The cytotoxic activity induced by the 3 different H-2D<sup>b</sup>-based FLK-1 minigenes was measured in this assay.

**Fig 6. Induction of IFN-γ at the intracellular and single T cell level.** (A) IFN-γ induced at the intracellular level was determined in splenocytes obtained 2 weeks after tumor cell challenge and by immunostaining with FITC-anti-CD4 or anti-CD8 Abs. Cells were fixed, permeabilized, and subsequently stained with PE-labeled anti-IFN-γ Abs to detect the intracellular expression of these cytokines. Production of IFN-γ was verified at the single-cell level by measuring production in individual T cells by the ELISPOT assay.

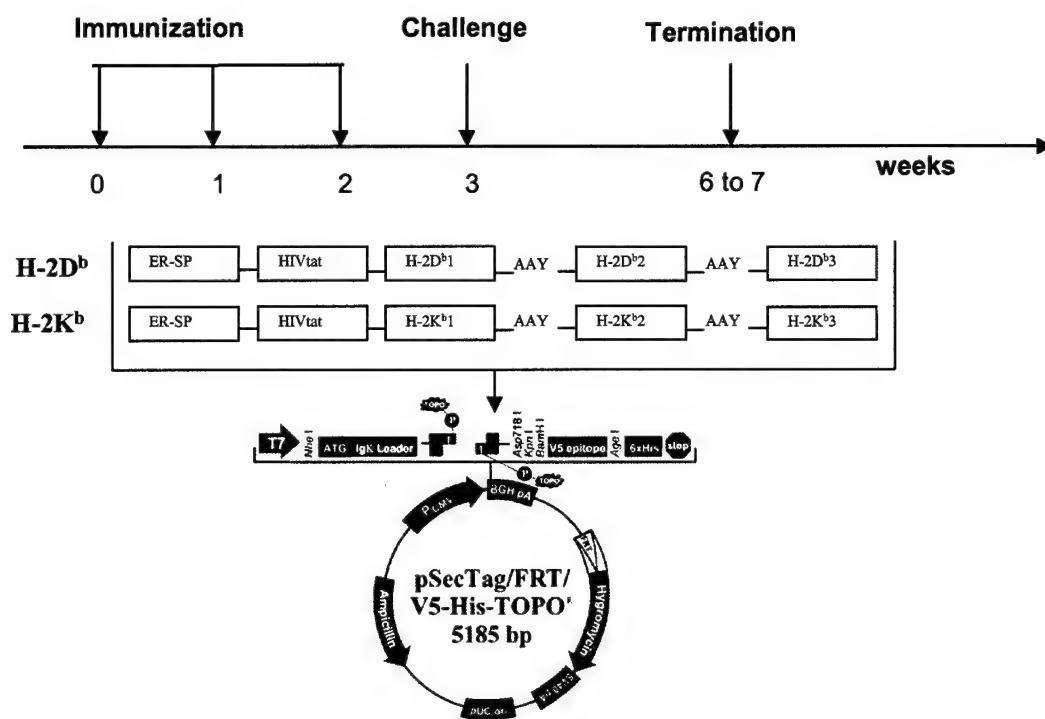


Fig. 1 Representative diagram for FLK-1 minigene vaccine strategy

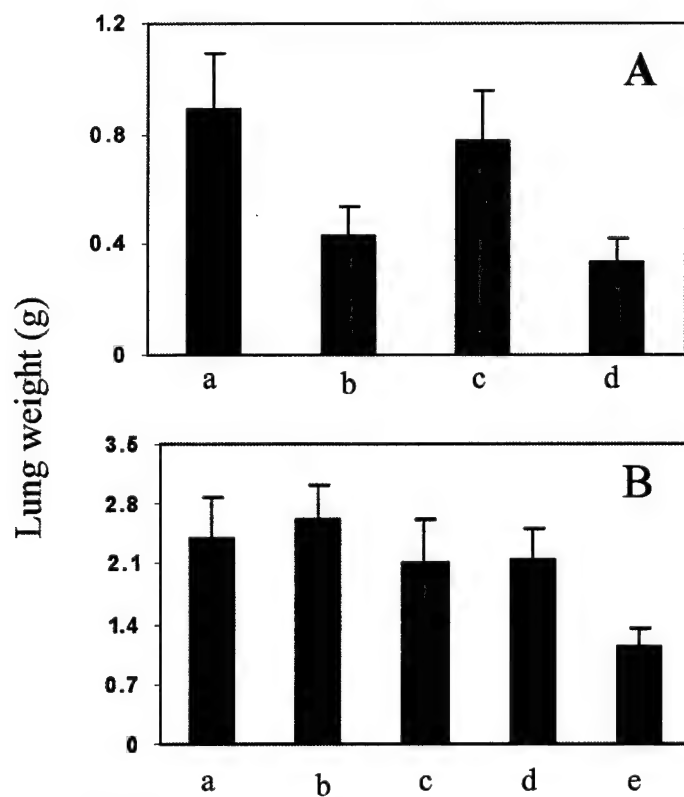


Fig. 2 Suppression of RM-9 prostate cancer growth by entire FLK-1 and FLK-1 minigene vaccines.

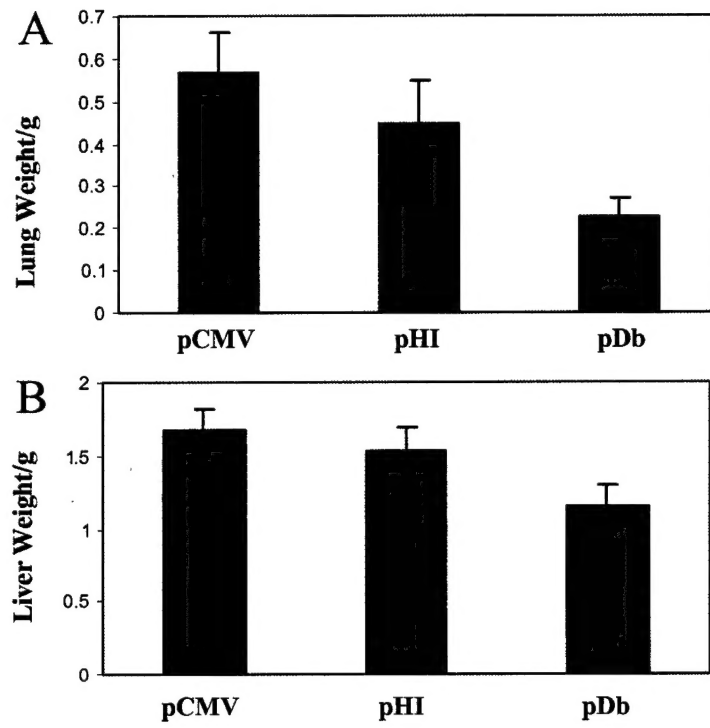


Fig. 3 Efficacy of H-2D<sup>b</sup> minigene vaccine against prostate cancer RM-9 in both lung and liver model

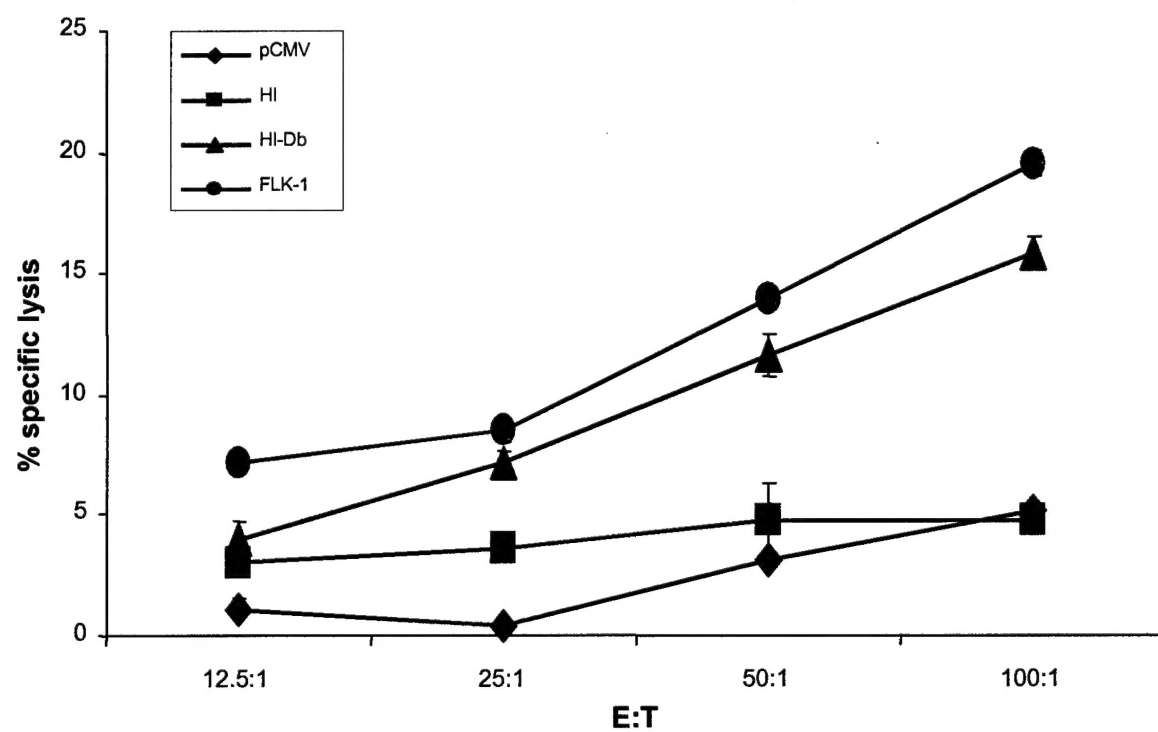


Fig. 4 T-cell mediated cytotoxicity against endothelial cell MS1 by FLK-1-based vaccine

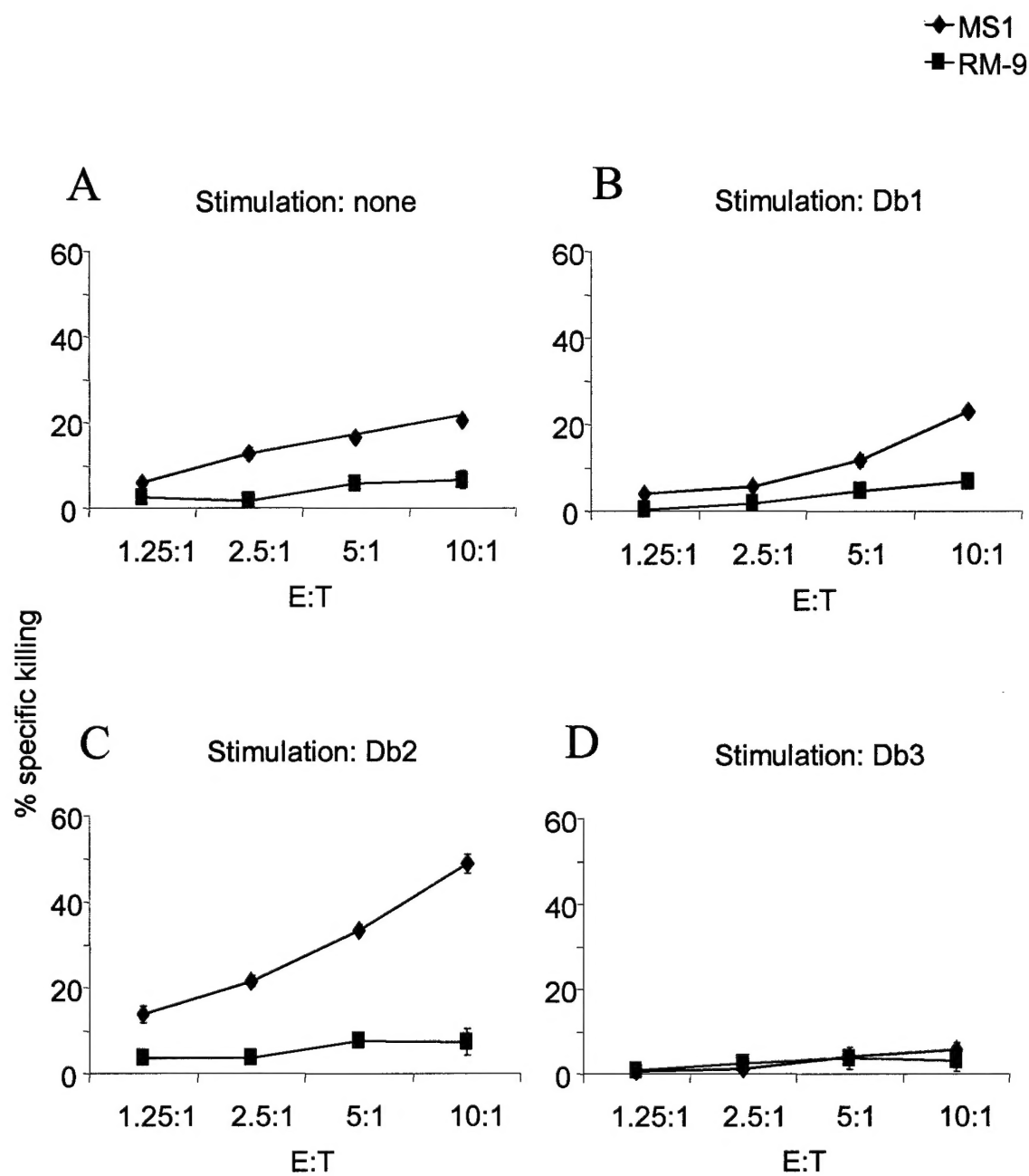


Fig. 5 T-cell induced cytotoxicity against endothelial and tumor cells by each H-2D<sup>b</sup> peptide



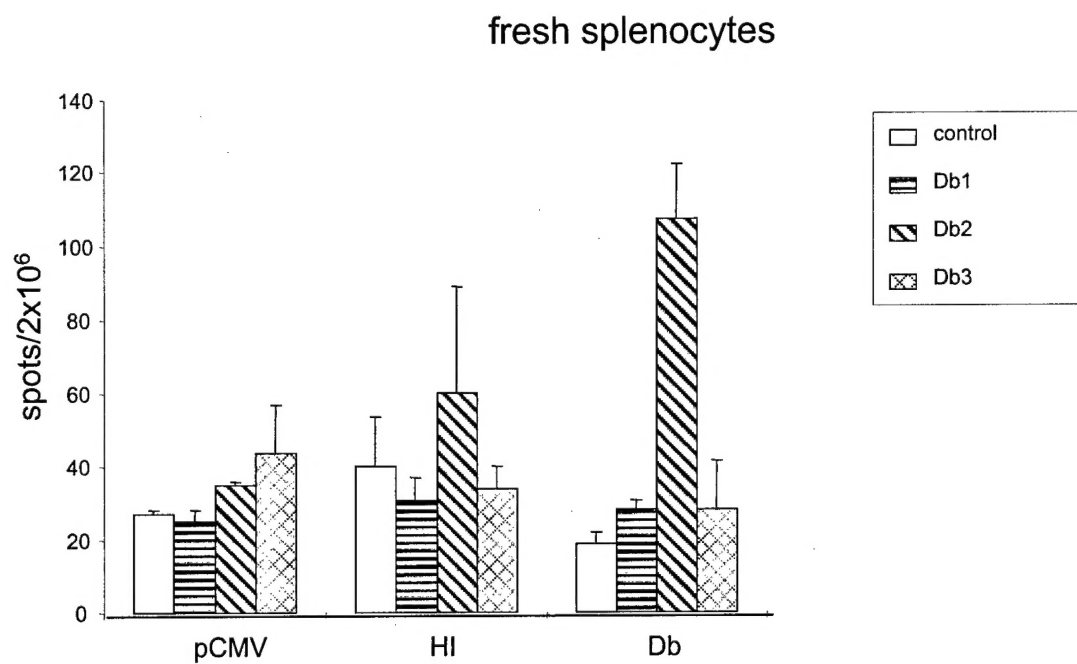


Fig. 6 Induction of Cytokines at the Intracellular and Single T cell Level by the individual H-2D<sup>b</sup> peptide